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Efficient Spore Synthesis in *Bacillus subtilis* Depends on the CcdA Protein

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CcdA is known to be required for the synthesis of *c*-type cytochromes in *Bacillus subtilis*, but the exact function of this membrane protein is not known. We show that CcdA also plays a role in spore synthesis. The expression of *ccdA* and the two downstream genes *yneI* and *yneJ* was analyzed. There is a promoter for each gene, but there is only one transcription terminator, located after the *yneJ* gene. The promoter for *ccdA* was found to be weak and was active mainly during the transition from exponential growth to stationary phase. The promoters for *yneI* and *yneJ* were both active in the exponential growth phase. The levels of the CcdA and YneJ proteins in the membrane were consistent with the observed promoter activities. The *ccdA* promoter activity was independent of whether the *ccdA-yneI-yneJ* gene products were absent or overproduced in the cell. It is shown that the four known cytochromes *c* in *B. subtilis* and the YneI and YneJ proteins are not required for sporulation. The combined data from analysis of sporulation-specific sigma factor activity, resistance properties of spores, and spore morphology indicate that CcdA deficiency affects stage V in sporulation. We conclude that CcdA, YneI, and YneJ are functionally unrelated proteins and that the role of CcdA in cytochrome *c* and spore synthesis probably relates to sulfhydryl redox chemistry on the outer surface of the cytoplasmic membrane.

The gram-positive, endospore-forming, bacterium *Bacillus subtilis* contains four different *c*-type cytochromes, which are all membrane anchored (2, 49, 52). The heme domain of these cytochromes is located on the outer surface of the cytoplasmic membrane. *B. subtilis* does not require cytochrome *c* for aerobic or anaerobic growth under laboratory conditions, and the physiological role of cytochromes of this type in the bacterium is not well understood.

The trademark of *c*-type cytochromes is that they contain protoheme IX covalently bound to the protein via thioether linkages (1, 29). Cytochrome *c* synthesis, i.e., the formation of the covalently bound heme, occurs on the outer (periplasmic) side of the cytoplasmic membrane in bacteria. In gram-negative bacteria, this biosynthetic process is assisted by several membrane-bound and periplasmic proteins (see reference 19, 27, and 44 for reviews). *B. subtilis ccdA*, *resB*, and *resC* are hitherto the only genes that have been shown experimentally to be required for cytochrome *c* synthesis in a gram-positive bacterium (21, 36). Genes encoding *B. subtilis* CcdA orthologues are present in members of the domains *Bacteria* (such as *Mycobacterium tuberculosis*, *Helicobacter pylori*, *Haemophilus influenzae*, *Treponema pallidum*, and cyanobacteria) and *Archaea* and also in plants (encoded by chloroplast genomes), but experimental data on the gene product in these organisms is not available. Very recently, CcdA was found in *Rhodobacter capsulatus* and shown to be involved in cytochrome *c* synthesis (8).

B. subtilis CcdA is an integral membrane protein of 228 or 235 amino acid residues (36). The exact function of this protein in the cell is not known, but it is required for a late step in the cytochrome *c* maturation pathway, after heme and apocytochrome have been transported across the cytoplasmic membrane (35). The amino acid sequence of CcdA is similar to that of the central part of DsbD (also named DipZ) of *Escherichia coli*, a protein of 489 residues which is thought to transfer reducing equivalents to disulfide isomerase(s) in the periplasm (6, 25, 31). The C-terminal part of DsbD, which has no similarity to CcdA, contains a thioredoxin-like sequence motif, -CysXaaYaaCys-. Cysteine residues in this motif and those that are invariant in DsbD and CcdA have been shown by site-specific mutagenesis to be functionally important (8, 39). It is notable that the genomes of some bacteria, for example *H. influenzae* and *M. tuberculosis*, contain genes for both CcdA and DsbD proteins whereas others, such as *E. coli* and *B. subtilis*, contain genes for only one of the two proteins (36). The *ccdA* gene, positioned at 164° on the *B. subtilis* chromosomal map, is cotranscribed with two downstream genes, *yneI* and *yneJ* (20) (originally named *orf120* and *orf160*, respectively [36]) (Fig. 1). YneI is most probably a single-domain response regulator. The sequence of this 120-residue protein is very similar to that of CheY and Spo0F, for which the three-dimensional structures are known (22, 40). YneJ is a predicted 160-residue integral membrane protein without clear similarity to any protein sequence available in the databases. YneI and YneJ are not required for cytochrome *c* biogenesis, and no clear difference in phenotype compared to the wild type has been observed with *yneI* or *yneJ* insertion mutants (36).

Sporulation in *B. subtilis*, i.e., the conversion of the vegetative cell into a spore, is a process characterized by ordered gene expression and complex morphological changes (10, 41). After the formation of an asymmetrically positioned septum between the mother cell and the forespore, the transition from one developmental stage to the next is governed by four sigma factors, σ^F and σ^G (forespore specific) and σ^E and σ^K (mother cell specific). Gene expression in the forespore and the mother cell is coordinated by intercompartment communication, where the appearance of an active sigma factor in one compartment is dependent on the activity of an earlier sigma factor in the other compartment. The end result is a spore much more resistant to heat and chemicals than is the vegetative cell.

To better understand the function of CcdA in *B. subtilis* and possibly find a role for the YneI and YneJ proteins in the cell, in this study we have analyzed the transcriptional organization of the *ccdA-yneI-yneJ* gene cluster and the expression of the

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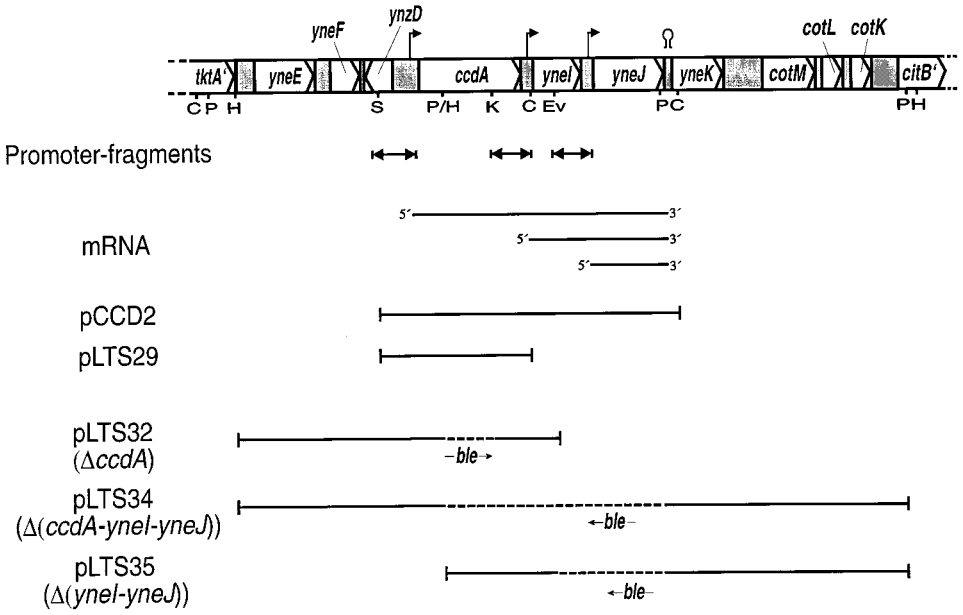

FIG. 1. Map of the *ccdA-yneI-yneJ* region in the wild-type *B. subtilis* chromosome. Transcription initiation sites, as indicated from primer extension analysis, and a termination site are indicated by hooked arrows and a hairpin symbol, respectively. DNA fragments cloned in pDG1728 and analyzed for promoter activity (double arrows) and mRNA species detected previously (36) by Northern blot analysis of strains containing plasmid pCCD2 are also shown. DNAs contained in plasmids pCCD2 and pLTS29, used in complementation studies, and plasmids pLTS32, pLTS34, and pLTS35, used in the construction of strains with deletions, are indicated by bars. The dashed lines in the latter DNAs indicate an approximately 1-kb fragment (not drawn to scale) harboring the *ble* resistance gene (an arrow indicates the relative orientation of the gene). Restriction enzyme cleavage sites: C, *Cla*I; E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sac*I.

three genes during growth. We demonstrate that CcdA is an integral membrane protein whose cellular concentration increases at the transition from exponential growth to stationary phase. Strains with *ccdA* deleted were found to be deficient, but not completely blocked, in the synthesis of spores with normal properties. This defect in sporulation was investigated.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. subtilis* strains and plasmids used in this work are presented in Table 1. *E. coli* strains JM83 [*ara* Δ(*lac-proAB*) *strA thi-1* (φ80 *lacZΔM15*)] (50), MM294 (*supE44 hsdR endA1 thi*) (30), and XL1-Blue [*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac* F′ *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10* (Tet^r)] (4) were used for the propagation of plasmids.

Media and general growth of bacteria. *E. coli* strains were grown on Luria agar plates or in Luria broth medium (34). *B. subtilis* strains were grown on tryptose blood agar base (TBAB) plates (Difco), Difco sporulation (DS) medium (16) [0.8% (wt/vol) Bacto nutrient broth (Difco), 0.1% (wt/vol) KCl, 0.012% (wt/vol) $MgSO_4 \cdot 7H_2O$, 0.5 mM NaOH, 1 mM $Ca(NO_3)_2$, 10 μM $MnCl_2$, 1 μM $FeSO_4$) plates, or Spizizen minimal medium (38) plates supplemented with required growth factors (10 mg/liter) and with 0.5% (wt/vol) glucose, succinate, or lactate as the carbon source. Nutrient sporulation medium with phosphate (NSMP) (11) or DS medium was used for liquid cultures, which were grown at 37°C in Erlenmeyer glass flasks (culture volume, ≤1/10 the volume of the flask) with indentations, on a rotary shaker at 200 rpm. For detection of β-galactosidase activity on TBAB plates, 80 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal) per liter was included in the medium. The following antibiotics were used when appropriate: ampicillin, 75 mg/liter; chloramphenicol, 12.5 mg/liter (*E. coli*) or 3 to 5 mg/liter (*B. subtilis*); erythromycin, 5 mg/liter; neomycin, 5 mg/liter; phleomycin, 1.2 mg/liter; spectinomycin, 150 mg/liter; and tetracycline, 25 mg/liter (*B. subtilis*).

Construction of plasmids and deletion mutants. Plasmid pLTS29, containing the *ccdA* gene, was constructed by first moving the 1.35-kbp *Sac*I-*Eco*RI fragment from pLTS17 to pUC18. The 1.12-kbp *Sal*I-*Cla*I fragment from the resulting plasmid, pLTS28, was isolated, and the ends of the fragment were made blunt by treatment with Klenow enzyme in the presence of deoxynucleoside triphosphates. Finally, the fragment was ligated to pHP13 cleaved with *Sma*I.

pLTS100, a high-copy-number plasmid carrying the *ccdA* gene, was constructed by moving the 1.2-kb *Eco*RI-*Bam*HI fragment of pLTS29 to pDGV1.

Plasmid pLTS33 was constructed by cloning the 1.7-kbp *Pst*I fragment of pMR22, which contains the region downstream of the *ccdA-yneI-yneJ* gene cluster and includes only the last 26 nucleotides of *yneJ*, into pBLE-1. A plasmid with the *Pst*I fragment in an orientation such that the *yneJ* end is close to the *ble* gene (encoding phleomycin resistance) was selected. Plasmids pLTS34 and pLTS35, used to delete the *ccdA-yneI-yneJ* and *yneI-yneJ* genes, respectively, were then constructed as follows. (i) The 1.52-kbp *Hin*dIII fragment of pLTS1 was moved to pBluescript SK(−) in an orientation such that the *ccdA′* part of the fragment is close to the *Kpn*I site in the cloning cassette. The 1.56-kbp *Eco*RI-*Kpn*I fragment from was then moved to pLTS33, resulting in plasmid pLTS34. (ii) The 844-bp *Hin*dIII-*Eco*RV fragment of pLTS17 was isolated and treated with Klenow enzyme in the presence of deoxynucleoside triphosphates. Subsequently, it was ligated with pBluescript SK(−) cleaved with *Sma*I (the insert was oriented such that the *yneI′* part of the fragment is close to the *Sac*I site in the cloning cassette). The 902-bp *Eco*RI-*Sac*I fragment from pLTS26 was then moved to pLTS33, resulting in plasmid pLTS35.

Plasmids pLTS34 and pLTS35 were linearized by cleavage with *Sca*I (which has a unique site in the *bla* gene) and used to transform *B. subtilis* 1A1 to phleomycin resistance. The resulting deletions and replacement with the *ble* gene were confirmed by Southern blot analysis. Note that the *ble* gene in previously reported deletion mutants obtained using pLTS32 (36) is in the opposite orientation to that in strains obtained from transformation with pLTS34 or pLTS35 (Fig. 1).

Strain LUT36 was constructed by subsequent transformations using linearized pΔcccA1 (to delete the *cccA* gene) (48) and chromosomal DNA from SL6820 (Δ*qcrC*::*neo*), JO1 (Δ*ctaCD*::*ble*), and L16205 (Δ*cccB*::*tet*) and selecting for the respective antibiotic resistance.

Construction of *ccdA-lacZ*, *yneI-lacZ*, and *yneJ-lacZ* transcriptional fusions. The regions upstream of *ccdA* (bp 1367 to 1682), *yneI* (bp 2257 to 2484), and *yneJ* (bp 2694 to 2920) were amplified by PCR using pCCD2 as template (Fig. 1). The *ccdA* region was amplified using *Taq* DNA polymerase (Roche Molecular Biochemicals) and the primer pair 5′ CGGAATTCCTGACTGAGCTCTATCG plus 5′ CGGGATCCATGATTTGACATTCCTTCAAG. The *yneI* and *yneJ* regions were amplified using *Pwo* DNA polymerase (Roche Molecular Biochemicals), and the primer pairs 5′-CGGAATTCTGAAGTGGATAAGGAAGAAC plus 5′-CGGGATCCAACAATCGATTTCCACAG and 5′-CGGAATTCTTAA CCTTTGATCCTAAAGC plus 5′-CGGGATCCGGAGTGTTGATACTATAT AC, respectively. *Eco*RI and *Bam*HI restriction sites were added via the primers (underlined). The amplified fragments were cut with *Eco*RI and *Bam*HI and ligated into pBluescript SK(−) or KS(−). The complete sequence of each cloned DNA fragment was determined to exclude errors introduced by the PCR. The *Eco*RI and *Bam*HI fragments were then moved to plasmid pDG1728, resulting in plasmids pLJJ7, pLJJ8, and pLJJ9 (Table 1). The *lacZ* gene of pDG1728 contains the Shine-Dalgarno sequence of the *B. subtilis spoVG* gene to provide efficient translation of the *lacZ* gene in *B. subtilis*. The obtained plasmids and pDG1728, as a negative control, were linearized by digestion with *Sca*I and

TABLE 1. *B. subtilis* strains and plasmids used in this work

Strain or plasmid	Description[a]	Source[b] or reference
Strains		
3G18	*ade met trpC2*	G. Venema
1A1	*trpC2*	BGSC[c]
1A1-J7	*trpC2* Δ*amyE*::(*ccdA′-lacZ spc*) Sp^r	This work
1A1-J8	*trpC2* Δ*amyE*::(*yneI′-lacZ spc*) Sp^r	This work
1A1-J9	*trpC2* Δ*amyE*::(*yneJ′-lacZ spc*) Sp^r	This work
1A1-DG	*trpC2* Δ*amyE*::(*lacZ spc*) Sp^r	This work
AD59	Ω*spoIID-lacZ* Cm^r	42
AD341	Ω*cotC-lacZ* Cm^r	53
AD454	*spoIIIG*::*neo* Nm^r	A. Driks
JO1	*ade met trpC2* Δ*ctaCD*::*ble* Pm^r	46
L16205	*trpC2* Δ*cccB*::*tet* Tc^r	2
LU60A1	*trpC2* Δ*ccdA*::*ble* Pm^r	LU6018→1A1
LU6018	*ade met trpC2* Δ*ccdA*::*ble* Pm^r	36
LU62A1	*trpC2* Δ(*ccdA-yneI-yneJ*)::*ble* Pm^r	pLTS34→1A1
LU62A1R$^{\#3}$	*trpC2* Δ(*ccdA-yneI-yneJ*)::*ble* (*ccdA* suppressor mutation-containing strain derived from LU62A1) Pm^r	This work
LU63A1	*trpC2* Δ(*yneI-yneJ*)::*ble* Pm^r	pLTS35→1A1
LU60A1-J7	*trpC2* Δ*ccdA*::*ble* Δ*amyE*::(*ccdA′-lacZ spc*) Sp^r	This work
LU62A1-J7	*trpC2* Δ(*ccdA-yneI-yneJ*)::*ble* Δ*amyE*::(*ccdA′-lacZ spc*) Sp^r	This work
LU63A1-J7	*trpC2* Δ(*yneI-yneJ*)::*ble* Δ*amyE*::(*ccdA′-lacZ spc*) Sp^r	This work
LU621	*trpC2* Ω*spoIID-lacZ* (Cm^r) Δ(*ccdA-yneI-yneJ*)::*ble* Pm^r	AD59→LU62A1
LU623	*trpC2* Ω*cotC-lacZ* Δ(*ccdA-yneI-yneJ*)::*ble* Cm^r Pm^r	AD341→LU62A1
LU624	*trpC2 spoIIIG*::*neo* Δ(*ccdA-yneI-yneJ*)::*ble* Nm^r Pm^r	AD454→LU62A1
LU625	*trpC2* Ω*sspB-lacZ* Δ(*ccdA-yneI-yneJ*)::*ble* Cm^r Pm^r	PM73→LU62A1
LU626	*trpC2* σ^F *spoIIIG*::*neo* Δ(*ccdA-yneI-yneJ*)::*ble* Em^r Nm^r Pm^r	PM73→LU624
LU627	*trpC2* σ^F *spoIIIG*::*neo sspB-lacZ* Δ(*ccdA-yneI-yneJ*)::*ble* Em^r Nm^r Cm^r Pm^r	PM73→LU626
LUA11	*trpC2* Ω*spoIID-lacZ* Cm^r	AD59→1A1
LUA13	*trpC2* Ω*cotC-lacZ* Cm^r	AD341→1A1
LUA14	*trpC2 spoIIIG*::*neo* Nm^r	AD454→1A1
LUA15	*trpC2* Ω*sspB-lacZ* Cm^r	PM73→1A1
LUA16	*trpC2* "σ^F" *spoIIIG*::*neo*, Em^r Nm^r	PM73→LUA14
LUA17	*trpC2* "σ^F" *spoIIIG*::*neo sspB-lacZ* Em^r Nm^r Cm^r	PM73→LUA16
LUT36	*trpC2* Δ*cccA*::*cat* Δ*cccB*::*tet* Δ*ctaCD*::*ble* Δ*qcrC*::*neo* Cm^r Tc^r Pm^r Nm^r	This work
PM73	*spoIIAC*Ω*spoIIAC* ("σ^F") *spoIIIG*Δ*1 sspB-lacZ Em^r Cm^r*	23
SL6820	*trpC2 lys-3 metB10* Δ*qcrC*::*neo Nm^r*	52
Plasmids		
pBLE-1	Ap^r Pm^r	12
pBluescript II KS(−), SK(−)	Ap^r	Stratagene
pCCD2	Cm^r Em^r; *ccdA-yneI-YneJ* on 2.1-kb fragment in pHP13	36 (Fig. 1)
pDGV1	Cm^r	3
pDG1728	Ap^r Sp^r; *B. subtilis amyE* deletion/integration vector with promoterless and modified *E. coli lacZ*	14
pHP13	Cm^r Em^r; *B. subtilis*/*E. coli* shuttle vector	15
pHV32	Ap^r Cm^r Tc^r	26
pLJJ7	Ap^r Sp^r; *316-bp ccdA* promoter fragment in pDG1728	This work
pLJJ8	Ap^r Sp^r; *228-bp yneI* promoter fragment in pDG1728	This work
pLJJ9	Ap^r Sp^r; *227-bp yneJ* promoter fragment in pDG1728	This work
pLTS1	Cm^r Em^r; *ccdA* on 2.7-kb fragment in pHP13	36
pLTS17	Ap^r; *ccdA* on a 2.7-kb fragment in pBluescript SK	36
pLTS29	Cm^r Em^r; *ccdA* on 1.1-kb fragment in pHP13	This work (Fig. 1)
pLTS32	Ap^r Pm^r; *ccdA* on 1.1-kb fragment in pHP13	36 (Fig. 1)
pLTS33	Ap^r Pm^r	This work
pLTS34	Ap^r Pm^r	This work (Fig. 1)
pLTS35	Ap^r Pm^r	This work (Fig. 1)
pLTS100	Cm^r; *ccdA* in pDGV1	This work
pMR22	Ap^r Tc^r	32
pUC18	Ap^r	50
pΔcccA1	Ap^r	48

[a] Ap^r, Cm^r, Em^r, Nm^r, Pm^r, Sp^r, and Tc^r indicate resistance to ampicillin, chloramphenicol, erythromycin, neomycin, phleomycin, spectinomycin, and tetracycline, respectively. "σ^F" indicates a mutant variant of the *spoIIAC* gene encoding a σ^F with a Val-233–to–Ala substitution.

[b] Arrows indicate transformation of the indicated strain with chromosomal or plasmid DNA.

[c] Bacillus Genetic Stock Center.

integrated, by means of transformation and a double-crossover recombination event, into the *amyE* locus of *B. subtilis* strains 1A1, LU60A1, LU62A1, and LU63A1. The desired spectinomycin-resistant transformants obtained were confirmed by erythromycin sensitivity and the lack of α-amylase activity and, in appropriate cases, also for defective cytochrome *c* synthesis by *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) oxidation staining.

Antisera and immunoblot analysis. Polyclonal antisera against CcdA and YneJ were obtained by immunizing New Zealand White rabbits with synthetic peptides conjugated to keyhole limpet hemocyanin. The peptides used, YITGV SMDDVKTEK and YRKLHNELQSSNIQMN, correspond to residues 32 to 45 of CcdA and 145 to 160 of YneJ, respectively. Custom peptide synthesis and production of antisera were carried out by Neosystems.

FIG. 2. Nucleotide sequence of promoter regions with transcription initiation sites (+1), as determined by primer extension analysis. Apparent σ^A-type -35 and -10 promoter sequences, translation start codons, and stop codons are indicated in bold type. There are two possible translation start codons for *ccdA*, as discussed previously (36). Putative ribosome binding sequences are underlined. The numbering of nucleotides is that used in reference 36.

For immunoblot analysis, cell extracts were incubated for 30 min at 40°C in the presence of 0.4% (wt/vol) sodium dodecyl sulfate (SDS), 0.2% (wt/vol) 2-mercaptoethanol, 1.2% (vol/vol) glycerol, and 5 mM Tris buffer (pH 6.8), and the proteins were then fractionated by SDS-polyacrylamide gel electrophoresis on 14% polyacrylamide gels by using the Schägger and von Jagow system (37). The proteins were transferred to Immobilon P membranes (Millipore) using 48 mM Tris–39 mM glycine buffer containing 20% (vol/vol) methanol and a semidry electroblot apparatus. Immunodetection was carried out by chemiluminescence using the ECL system (Pharmacia Amersham). Primary antisera were used at a 500-fold dilution and secondary antibodies, donkey anti-rabbit immunoglobulin G conjugated to horseradish peroxidase, were used at a 3,000-fold dilution.

β-Galactosidase activity measurements. To determine β-galactosidase activity in cells, growth medium was inoculated with cells from an exponentially growing culture. Samples were withdrawn at intervals during the growth of the batch culture, immediately frozen in liquid nitrogen, and then stored at −20°C. The samples were thawed in cold water, and the cells were collected by centrifugation for 1 min at 20,000 × *g* in an Eppendorf centrifuge. The cell pellets were suspended in AB buffer (60 mM K_2HPO_4, 40 mM KH_2PO_4, 100 mM NaCl). A 175-μl volume of a suspension of an appropriate cell concentration was mixed with 35 μl of 4-methylumbelliferyl-β-D-galactoside (MUG), 0.4 g/liter in dimethyl sulfoxide, and incubated at 23°C on a water bath. At intervals, 50-μl aliquots were withdrawn and diluted in 2.45 ml of AB buffer, and the fluorescence was immediately determined using a Shimadzu RF-5301PC spectrofluorometer with an excitation wavelength of 366 nm (3-nm slit) and an emission wavelength of 445 nm (10-nm slit). The fluorescence of solutions of 4-metylumbelliferone of known concentrations in AB buffer were used to determine the amount of MUG hydrolyzed in the samples. Rates of MUG hydrolysis were obtained from linear plots of product concentration versus time. Specific β-galactosidase activities were calculated for the cell concentration estimated from the optical density at 600 nm (OD_{600}) of the original sample taken from the culture.

Spore assays. Cultures were grown in 25 ml of NSMP (pH 6.5) at 30°C in a 250-ml Erlenmeyer flask for 2 days. The spore titer was determined by subjecting the cultures to heat, chloroform, or lysozyme treatment. Routinely, 5-ml samples were heated at 80°C for 10 min (unless stated otherwise) or vigorously mixed with 0.6 ml of chloroform for 10 s, or a small volume was diluted 100-fold in minimal salts solution [80.4 mM K_2HPO_4, 44.1 mM KH_2PO_4, 0.8 mM $MgSO_4$, 15.1 mM $(NH_4)_2SO_4$, 3 mM sodium citrate] and incubated with or without lysozyme (0.5 g/liter) at 30°C for 30 min. Serial dilutions were plated on TBAB plates and incubated overnight at 37°C. Dilutions of nontreated samples were plated in parallel.

Electron microscopy. Cells were grown in NSMP medium (pH 6.5) at 30°C in a 250-ml Erlenmeyer flask for 3 days. Samples (10 ml) were harvested and washed in fresh medium once. The cell pellet was treated with 2.5% glutaraldehyde, fixed in OsO_4, and dehydrated in ethyl alcohol, and the sample was finally impregnated in plastic and sections were analyzed by electron microscopy.

Other methods. *B. subtilis* chromosomal DNA was isolated by the method of Marmur (24). Plasmid DNA was isolated by using a kit (Maxi Prep [Qiagen] or Jet Prep Plasmid Miniprep [Genomed]). Total RNA from *B. subtilis* was purified using the RNeasy kit (Qiagen). Primer extension analysis was done as described previously (36) using oligonucleotides complementary to the proximal part of *yneI* (bp 2609 to 2593, 5′-CAGCTGCTTGTTCACCG) and *yneJ* (bp 3028 to 3012, 5′-AACAGGCTTGTCAGAGG) (base pair numbering refers to the published sequence [36], accession no. X87845). *E. coli* was transformed by electroporation and *B. subtilis* was grown to competence essentially as previously described (18). Oxidation of the artificial electron donor TMPD by colonies on TBAB plates was assayed by the soft-agar overlay method (13). α-Amylase production was assayed by growth on TBAB plates containing 1.5% (wt/vol) starch and then staining colonies with KI-I_2 solution. Membranes from *B. subtilis* strains were isolated from osmotically lysed cells essentially as described by Hederstedt (17) and stored at −80°C. Light absorption spectroscopy was carried out as described previously (36). The protein concentration of membrane fractions was determined using the bicinchoninic acid protein assay (Pierce Chemical Co.) with bovine serum albumin as the standard.

RESULTS

Promoters for the *yneI* and *yneJ* genes. By using Northern blot analysis, *ccdA-yneI-yneJ*, *yneI-yneJ*, and *yneJ* mRNAs have previously (36) been detected (Fig. 1). The *ccdA* gene is preceded by an apparent σ^A-type promoter, and the initiation site for transcription is positioned about 100 bp from the translational start codon (36). Plasmid pCCD2 is a low-copy-number plasmid containing the *ccdA-yneI-yneJ* gene cluster and flanking DNA (Fig. 1 and Table 1). Primer extension analysis, with total RNA extracted from *B. subtilis* strain 3G18/pCCD2 grown in NSMP, identified the 5′ end of the *yneI-yneJ* and *yneJ* mRNAs (data not shown and Fig. 2). The indicated transcription initiation sites are located in intergenic regions and are both positioned 33 bp from the translation start codon (ATG) for the respective gene. An apparent −10 sequence of a σ^A-

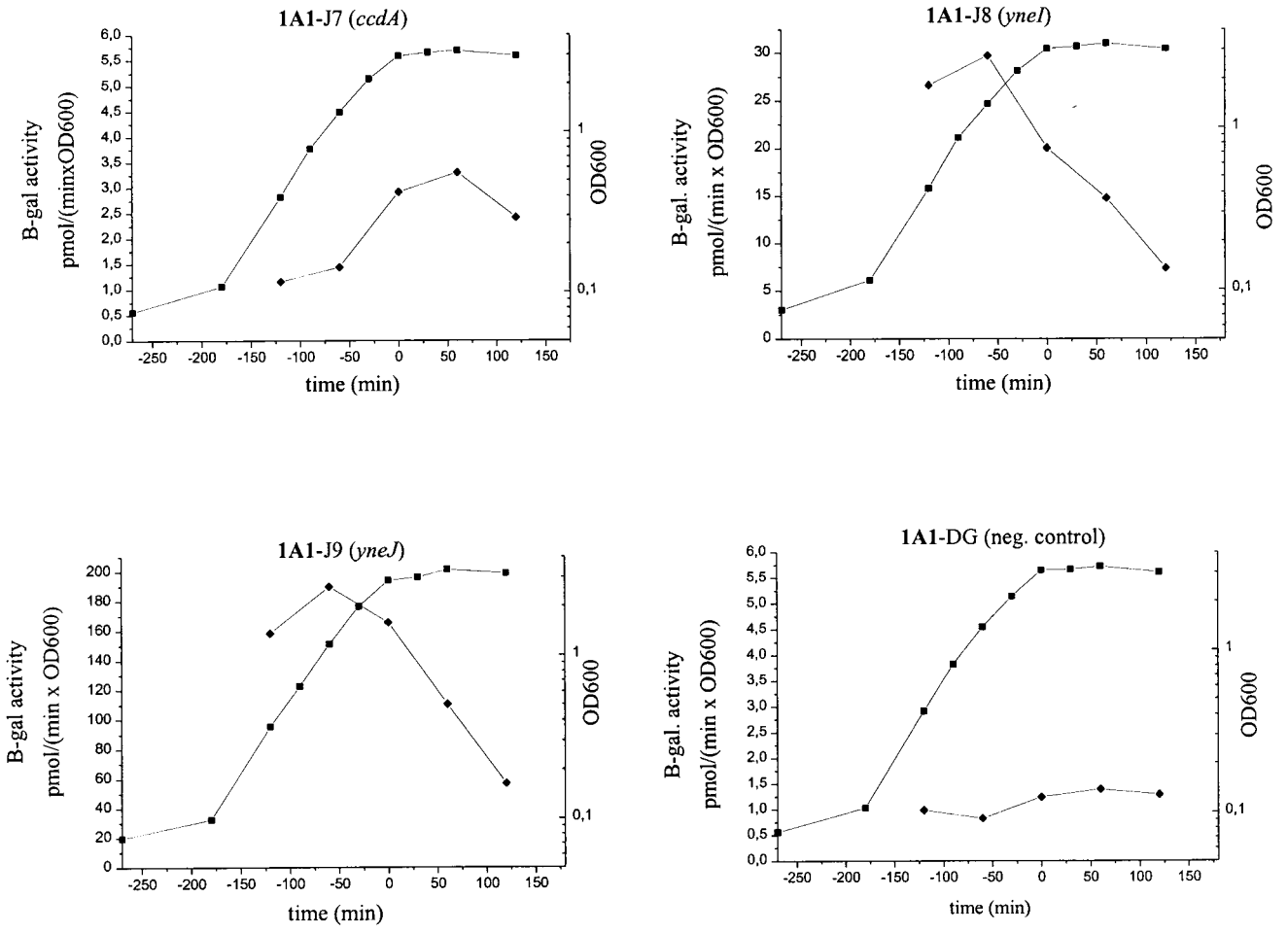

FIG. 3. β-Galactosidase activity of *B. subtilis* strains grown in NSMP. ■, OD_{600}; ◆, specific enzyme activity. Note the different scales for enzyme activity in the panels. The strains contain the *ccdA*, *yneI*, or *yneJ* promoters fused to a promoterless *lacZ* gene and integrated into the chromosome in one copy. See Table 1 for a full description of strains.

type promoter, but no corresponding −35 sequence, is present in the promoter regions of *yneI* and *yneJ* (Fig. 2).

Promoter activities during growth. DNA fragments predicted to contain the three promoter regions were amplified by PCR and cloned in front of the promoterless *lacZ* gene in plasmid pDG1728. The *ccdA*, *yneI*, and *yneJ* fragments contain 224, 202 and 215 bp, respectively, of sequence upstream of +1 and the leader sequence up to (but not including) the ribosome binding sequence (Fig. 1 and 2). Each *lacZ* transcriptional fusion was integrated in single copy into the *amyE* locus on the chromosome of *B. subtilis* 1A1 (parental strain), LU60A1 (Δ*ccdA*), LU62A1 [Δ(*ccdA-yneI-yneJ*)], and LU63A1 [Δ(*yneI-yneJ*)] (Table 1; see Materials and Methods for details).

To analyze the relative strength and temporal activity of each promoter during growth, strain 1A1 containing the three different *lacZ* fusions, i.e., strains 1A1-J7, 1A1-J8, and 1A1-J9, and the negative control, 1A1-DG, were grown in NSMP and the cells were assayed for β-galactosidase enzyme activity (Fig. 3). The results indicate that the *ccdA* promoter is weak (the maximal β-galactosidase activity was 3 pmol/min × OD_{600}) and active during the transition from exponential growth to stationary phase. In contrast, the *yneI* and *yneJ* promoters are active during exponential growth phase and gradually decrease in activity as the culture progresses into stationary phase. The maximal β-galactosidase activity obtained with the *yneJ-lacZ* gene fusion (190 pmol/min × OD_{600}) was about 9- and 60-fold higher than those obtained with the *yneI-lacZ* and *ccdA-lacZ* fusions, respectively.

The β-galactosidase activity profiles of strains with the entire *ccdA-yneI-yneJ* cluster deleted and containing the respective transcriptional fusion at the *amyE* locus were essentially the same as those obtained with the different *lacZ* fusions in the parental genetic background (data not shown). The β-galactosidase activity during growth was also analyzed for strains 1A1-J7, LU60A1-J7, LU62A1-J7, and LU63A1-J7, each harboring one copy of the *ccdA* promoter-*lacZ* fusion in the chromosome and containing pLTS1 (*ccdA*), pCCD2 (*ccdA-yneI-yneJ*), or pHP13 (plasmid vector). No significant differences in β-galactosidase activity or temporal pattern were observed with these 12 strains compared to strain 1A1-7J (Fig. 3 and data not shown). The combined data strongly suggest that gene products of the *ccdA-yneI-yneJ* cluster do not regulate the transcription of *ccdA*. This is based on the assumption that the 316-bp fragment used for promoter activity analysis comprises the entire promoter region with potential regulatory elements.

CcdA and YneJ protein profiles. The subcellular localization and the steady-state levels of CcdA and YneJ in *B. subtilis* were analyzed using immunoblot with antisera against oligopeptides corresponding to amino acid residues 32 to 45 of CcdA (assuming ATG to be the start codon [Fig. 2]) and the C-terminal

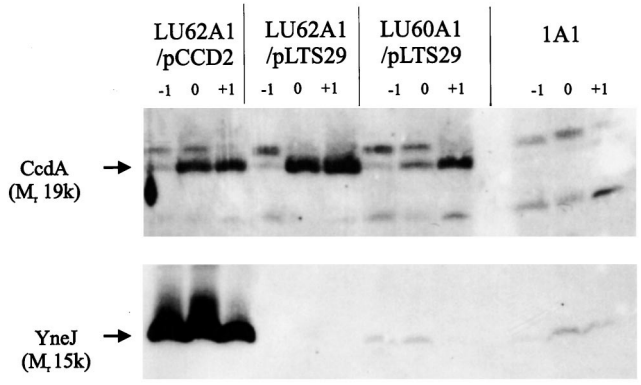

FIG. 4. Immunoblot analysis of membrane proteins from different *B. subtilis* strains, using CcdA antiserum (upper panel) and YneJ antiserum (lower panel). The blots are overexposed to demonstrate the presence of small amounts of CcdA and YneJ antigen. Strain LU62A1 has the *ccdA-yneI-yneJ* gene cluster deleted, LU60A1 has *ccdA* deleted, and 1A1 is the parental strain. Plasmids pLTS29 and pCCD2 carry the *ccdA* gene and the *ccdA-yneI-yneJ* gene cluster, respectively. The designations −1, 0, and +1 indicate that membranes were isolated from cells harvested 1 h before, at the time, and 1 h after exponential growth ended, respectively. The bands in the top panel seen just above CcdA and in the lower part of the gel are due to cross-reactivity with unrelated antigens and were apparent only on overexposed films.

16 residues of YneJ, respectively. Extracts were prepared from cells grown in NSMP and harvested 1 h before the end of the exponential growth phase (T_1), at the end of the exponential growth phase (T_0), and 1 h into the stationary phase (T_{+1}), respectively. Typical growth curves are shown in Fig. 3.

A CcdA antigen of 19 kDa and a YneJ antigen of 15 kDa were found in isolated membranes from strain LU62A1/pCCD2 (Fig. 4). As expected, these antigens were not present in membranes of strain LU62A1/pHP13, which lacks the *ccdA* and *yneJ* genes (immunoblot not shown). The molecular sizes of the polypeptide antigens as deduced from the SDS-polyacrylamide gels were smaller than those calculated from the DNA sequence, 25 or 26 kDa for CcdA (depending on the translational initiation codon used [Fig. 2]) and 18.3 kDa for YneJ. Such deviations in apparent size are often seen for integral membrane proteins. The CcdA protein could be extracted from the membrane with nonionic detergent, consistent with it being an integral membrane protein.

Even after overexposure of the immunoblot, CcdA antigen was not detected in membranes from strain 1A1, which contains a single copy of the *ccdA* gene in the chromosome. The protein was found only in strains containing *ccdA* on a plasmid, e.g., pCCD2 and pLTS29 (Fig. 4, upper panel). YneJ antigen was found in membranes of both strain 1A1 and the *ccdA* deletion mutant LU60A1 (lower panel). The amount of CcdA polypeptide was small in membranes from exponentially growing cells compared to that in membranes from cells in a later growth stage. YneJ showed a different pattern, with larger amounts present in membranes from exponentially growing cells compared to stationary-phase cells. Thus, the observed variations in cellular amounts of CcdA and YneJ protein are consistent with those in mRNA levels as determined by Northern blot analysis (36) and in activity patterns observed with the *ccdA*, *yneI*, and *yneJ* promoter regions fused to *lacZ* (Fig. 3).

Phenotype of strains deficient in *ccdA*, *yneI*, and *yneJ*. *B. subtilis* strains derived from 1A1 and containing deletions in the *ccdA*, *yneI*, and *yneJ* gene cluster (Fig. 1) were tested for growth on TBAB and minimal plates with glucose, succinate, or lactate as the carbon source. No major differences in growth were seen between the various mutants and the parental strain after incubation at 37°C overnight. Incubation of colonies on TBAB or minimal-succinate plates at room temperature for 2 days, however, resulted in lysis of LU60A1 (Δ*ccdA*) and LU62AI [Δ(*ccdA-yneI-yneJ*)]. Colonies of strains 1A1 and LU63A1 [Δ(*yneI-yneJ*)] did not lyse on the plates even after >1 week of incubation at room temperature.

TABLE 2. Sporulation efficiency of *B. subtilis* strains carrying deletions in the *ccdA-yneI-yneJ* region

Strain	Relevant genotype	Total-cell titer[a] (CFU/ml)	Spore titer[b] (CFU/ml)	Sporulation efficiency[c] (%)
1A1	Wild type	8.7×10^8	7.8×10^8	90
LU60A1	Δ*ccdA*	1.4×10^8	5.2×10^6	3.7
LU62A1	Δ(*ccdA-yneI-yneJ*)	2.0×10^8	2.7×10^6	1.4
LU63A1	Δ(*yneI-yneJ*)	7.6×10^8	6.6×10^8	87

[a] Titer is CFU per milliliter of culture after growth for 2 days at 30°C in NSMP.
[b] Titer after incubation of the sample at 75°C for 10 min.
[c] Sporulation efficiency is calculated as spore titer divided by total cell titer, multiplied by 100.

Growth of 1A1 and the derivatives with various deletions in the *ccdA-yneI-yneJ* region was also tested in NSMP and DS liquid medium. No significant differences in exponential growth rate were observed between the strains, and they reached the same OD at the end of exponential growth phase. A few hours into the stationary phase, the optical density of cultures of strains LU60A1 and LU62A1, both with the *ccdA* gene deleted, progressively decreased, and this was correlated with a decrease in the number of CFU (data not shown and Table 2). These growth properties of the mutant strains indicated that CcdA deficiency affects sporulation.

CcdA-deficient mutants show reduced sporulation efficiency. The ability of *ccdA* mutant strains to form spores was analyzed by growing cultures in NSMP for 2 days at 30°C and then determining the fraction of heat- and chloroform-resistant cells in the cultures (Tables 2 and 3). For the parental strain, 1A1, and the *yneI-yneJ* deletion mutant, LU63A1, the sporulation efficiency was >85%. Strains LU60A1 and LU62A1, with *ccdA* and *ccdA-yneI-yneJ*, respectively, deleted, showed a sporulation efficiency of 1 to 6%. This number is much greater than that obtained with a strain completely blocked in an early step in sporulation such as the SpoIIIG-deficient strain LUA14 (Table 3). The spore yields per volume of culture were very low for the CcdA-deficient strains compared to the parental strain

TABLE 3. Heat, chloroform, and lysozyme resistance of cells grown for sporulation in NSMP

Strain	Relevant genotype	Treatment[a]	Cell titer before treatment (CFU/ml)	Survival[b] (%)
1A1	Wild type	Heat	4.1×10^8	92
		Chloroform	4.1×10^8	89
		Lysozyme	4.1×10^8	85
LU62A1	Δ(*ccdA-yneI-yneJ*)	Heat	0.62×10^8	2.6
		Chloroform	0.62×10^8	5.6
		Lysozyme	0.62×10^8	18
LUA14	*spoIIIG*	Heat	2.4×10^8	<0.005
		Chloroform	2.4×10^8	<0.005
		Lysozyme	2.4×10^8	2.8

[a] See Materials and Methods for details.
[b] Survival is CFU per milliliter after treatment divided by that before treatment, multiplied by 100.

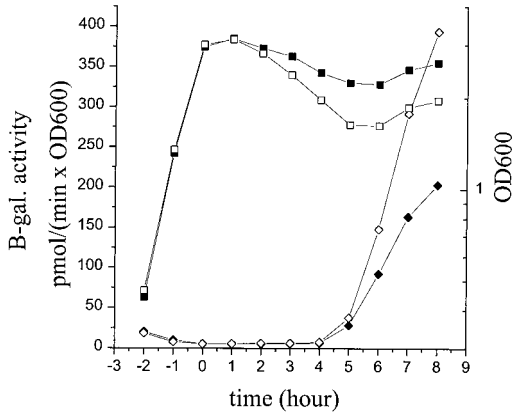

FIG. 5. σ^K-dependent expression of a *cotC-lacZ* gene fusion in the chromosome of the wild-type control strain LUA13 (solid diamonds) and CcdA-deficient strain LU623 (open diamonds) during growth in DS medium. The square symbols show OD_{600} (solid, LUA13; open, LU623). Time zero is the end of the exponential growth phase.

because of the cell lysis of the former strains that occurs in the stationary phase. Incubation of spore-containing cultures at 80°C for 10, 15, and 30 min did not reveal any difference in heat sensitivity between spores of strain 1A1 and LU62A1 (data not shown). The results indicate that CcdA-deficient mutants can synthesize spores with normal properties at only a low efficiency.

Clones from germinated spores of strains LU60A1 and LU62A1, i.e., cells from colonies obtained on TBAB plates after heat treatment of spore-containing cultures, showed the same reduced sporulation efficiency as LU60A1 and LU62A1. This demonstrated that the heat-resistant spores obtained from cultures of CcdA-defective strains are not due to revertants in the cultures.

B. subtilis wild-type spores are also resistant to lysozyme treatment. Strains with the *ccdA* gene deleted showed an intermediate sensitivity to lysozyme compared to the parental strain and strain LUA14 blocked at stage III in sporulation (Table 3).

Phase-contrast light microscopy of cultures of LU60A1 and LU62A1, grown for sporulation, showed only a few light-diffracting cells in contrast to corresponding cultures of 1A1, which contained such cells predominantly. Cells in cultures of the CcdA-deficient strains were mostly nonmotile. Thus, it seems as if many cells in a culture of a CcdA-deficient strain can progress in spore synthesis up to, but not beyond, a relatively lysozyme-resistant state where dehydration occurs in the wild-type case, yielding light-diffracting spore structures. A small percentage of the sporulating CcdA-deficient cells apparently are able to complete the differentiation process and mature into strongly light-diffracting heat-resistant spores.

Sporulation-specific sigma factor activity in a CcdA-deficient mutant. To determine if CcdA is important for the sporulation-specific sigma factor cascade, we analyzed the expression of different promoter-*lacZ* fusions integrated as a single copy into the *amyE* locus of *B. subtilis* strains with and without an intact *ccdA* gene. The *spoIID*, *sspB*, and *cotC* promoters in the transcriptional fusions we used are dependent on σ^E, σ^G, and σ^K, respectively, for activity. σ^F-dependent gene expression was analyzed by the use of strains LUA17 and LU627, which do not synthesize σ^G and produce a variant of σ^F that acts like σ^G, mediating transcription of the *sspB* promoter-*lacZ* fusion.

All sporulation-specific sigma factors were found to be active in both the parental genetic background (strains LUA11, LUA13, LUA15, and LUA17) and the strains (LU621, LU623, LU625, and LU627) lacking the *ccdA* gene, as assayed by the blue color of colonies on TBAB plates supplemented with X-Gal. In liquid NSMP cultures, the expression of the σ^E-, σ^F-, and σ^G-dependent *lacZ* gene fusions, as determined by β-galactosidase activity, was about the same and appeared at the same time point in the growth curve in the CcdA-defective strains as in the control strains (data not shown). The σ^K-dependent gene fusion was expressed to a somewhat higher level in the CcdA-deficient strain LU623 than in strain LUA13 (Fig. 5). It is uncertain whether this observed reproducible minor difference is relevant for a better understanding of the function of CcdA in the cell. Since the expression of the *cotC-lacZ* fusion is dependent on both σ^K and GerE activity (the latest-acting sporulation-related transcription factor) GerE must also be active in a CcdA-defective mutant (41).

Morphology of mutant spores. Electron microscopy of samples from sporulating cultures of strain 1A1 and LU62A1 showed more cell lysis in the latter strain. Spore-like structures, often enclosed by the mother cell, were seen in samples of both strains (Fig. 6 and electron micrographs not shown). Those of LU62A1, corresponding to the nonmotile cells seen by light microscopy, showed the main features of *B. subtilis* spores, i.e., a cortex, a lamellar inner coat, and an electron-dense outer coat. We could not identify a consistent structural difference in the morphology of the spores of 1A1 and LU62A1, except that the cytoplasm of the latter strain appeared more granular. From the sporulation efficiencies of the strains (Tables 2 and

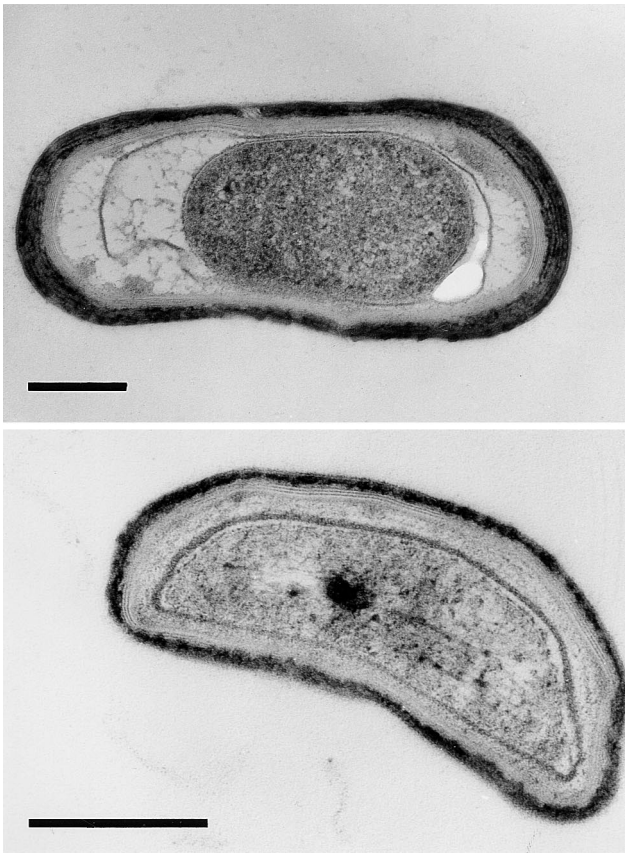

FIG. 6. Electron micrographs of two representative *B. subtilis* LU62A1 cells from a culture grown for sporulation. Bars, 0.4 μm.

3), less than 10% of the LU62A1 spores observed by electron microscopy would be heat-resistant cells. Judging from the relative lysozyme resistance, the poor light diffraction properties, and the heat and chloroform sensitivity of most spores of CcdA-deficient mutants, we consider it likely that synthesis of the cortex and/or coat and dehydration of the spore are defective.

Complementation of sporulation defects by the *ccdA* gene on plasmids. The deficiency in spore synthesis of strains LU60A1 and LU62A1 was complemented by the *ccdA* gene or the *ccdA-yneI-yneJ* gene cluster on plasmids, i.e., by pLTS29 and pCCD2 (data not shown). These plasmids also restored cytochrome *c* synthesis, as determined by the ability of colonies on TBAB plates to oxidize TMPD. Plasmid pLTS100, a high-copy-number plasmid carrying the *ccdA* gene, complemented the defect in cytochrome *c* synthesis in strain LU60A1 and caused a ca. fivefold overproduction of CcdA protein compared to that in LU62A1/pCCD2 (immunoblot not shown). In contrast to strains containing the low-copy-number plasmids pLTS29 or pCCD2 or the vector pGDV1, those containing pLTS100 gave rise to small colonies and were unstable. We do not know if this apparent toxicity of pLTS100 is due to functional activity of CcdA or physical disturbance of the membrane caused by the increased amounts of this integral membrane protein.

A *B. subtilis* strain with a nonsense mutation in the *ccdA* gene has recently been isolated and characterized (21). The phenotype of this strain is identical in all aspects to that of LU60A1, with *ccdA* deleted. This, together with the result of the complementation experiments and the properties of a strain deficient in translation of *ccdA* (36), demonstrates that the CcdA protein is required for both efficient sporulation and cytochrome *c* synthesis in *B. subtilis*.

Isolation of strains with suppressor mutations. Microcolonies (papilla) appeared within lysed colonies of strain LU62A1 on TBAB plates incubated at room temperature for >1 week. Cells from these microcolonies formed normal-sized colonies when streaked on TBAB plates and oxidized TMPD, albeit more poorly than did 1A1 colonies. These clones were also phleomycin resistant and contained the same *ccdA-yneI-yneJ*::*ble* deletion-substitution as in strain LU62A1, as determined by Southern blot analysis of chromosomal DNA. Thus, the clones contain suppressor mutations. The suppressor-containing strains showed wild-type sporulation efficiency (80 to 90%), and light absorption spectroscopy of membranes isolated from one clone, LU62A1R$^{\#3}$, confirmed the presence of *c*-type cytochromes (spectra not shown). Strain LU62A1R$^{\#3}$ and other suppressor-containing strains differ from LU62A1 in that they do not develop competence. The suppressor mutations can be moved to other strains by transformation and are not linked to the *ccdA* locus on the chromosome. The results show that the suppressor mutation(s) at one or several loci can restore all known cell defects caused by CcdA deficiency. Despite several attempts, using different strategies and isolates, we have so far not been able to identify any of the suppressor mutations.

***c*-type cytochromes are not required for sporulation.** The four *c*-type cytochromes in *B. subtilis*, cytochrome c_{550}, cytochrome c_{551}, the cytochrome *c* subunit of the *bc* complex, and subunit II of the cytochrome caa_3 oxidase, are encoded by the *cccA*, *cccB*, *qcrC*, and *ctaC* genes, respectively (2, 49, 52). Respiration-defective *B. subtilis* mutants are generally sporulation deficient (43). Defective sporulation of CcdA-deficient strains could therefore be a result of the total lack of cytochrome *c*. To determine this, we constructed strain LUT36, which has the structural genes for all four cytochromes *c* deleted. LUT36 was found to have close to normal sporulation efficiency (>60%), demonstrating that cytochrome *c* is not important for sporulation to occur in *B. subtilis*.

DISCUSSION

In this work we demonstrate that sporulation in *B. subtilis* can occur in the absence of the four known *c*-type cytochromes and that the *ccdA* gene is required for efficient sporulation. Our available experimental data, taken together, strongly suggest that CcdA deficiency affects a late step in spore synthesis, probably synthesis of the cortex and/or coat and dehydration of the spore.

The *ccdA* gene is cotranscribed with the *yneI* and *yneJ* genes from a promoter upstream of *ccdA* (36), and in this work we demonstrate that there are also promoters for the synthesis of *yneI-yneJ* and *yneJ* mRNAs. These last two promoters show a different temporal pattern of activity during growth of batch cultures compared to the promoter for *ccdA*. The expression from the *ccdA* promoter is not regulated by CcdA, YneI, or YneJ, since the absence or overproduction of these three proteins did not affect *ccdA* promoter activity. Genes for proteins that physically interact are in bacteria often clustered and present in a conserved order on the chromosome (7). *B. subtilis* genes for functionally closely related proteins, for example enzymes of a biosynthetic pathway, are generally arranged in operons (20). There is, however, no evidence suggesting that the function of the YneI or YneJ protein in the cell is related to that of CcdA. Strains lacking or overexpressing *yneI* and *yneJ* are proficient in cytochrome *c* and spore synthesis. Moreover, the *ccdA* gene in other gram-positive bacteria of known genome sequence (5) is not flanked by genes corresponding to *yneI* or *yneJ*. The *B. subtilis cheY* and *spo0F* genes encode single-domain response regulators very similar to YneI, but neither of these genes are flanked by a gene for a membrane protein like YneJ. Although transcription of *ccdA*, *yneI*, and *yneJ* rely on a common terminator located after the *yneJ* gene, we conclude that the three genes probably encode functionally unrelated proteins. The transcriptional organization of the gene cluster does not cause a problem in the cell, because the promoters in front of the genes progressively increase in strength in steps of approximately 1 order of magnitude.

Cytochromes of the *c* type are present in exponentially growing *B. subtilis* cells and increase in concentration together with other cytochromes at the end of exponential growth (45). Transcription of structural genes for *c*-type cytochromes and *ccdA* also increases at this growth phase or at the entry into stationary phase in NSMP medium (2, 51). Exponentially growing cells are expected to contain some CcdA protein, since cytochrome *c* is present. It might be of functional importance to maximize the expression of *ccdA* when the cell approaches stationary phase, since CcdA is needed for efficient spore synthesis.

The hydrophobicity profile, combined with sequence comparisons and application of the positive-inside rule (47) and topology studies using protein fusions (8, 39), indicates that the CcdA protein has six α-helical transmembrane segments and the C-terminal end exposed on the outer side of the cytoplasmic membrane. Two cysteine residues, located far apart in the primary sequence, are conserved in CcdA sequences (references 8 and 36 and our unpublished data) and are functionally important (8). The amino acid sequence similarity between *B. subtilis* CcdA and *E. coli* DsbD and the importance of both proteins for a late step in cytochrome *c* synthesis (6, 33, 35) suggest that CcdA, together with a thioredoxin-like protein, has a function similar to that of DsbD in the transfer of reducing equivalents across the cytoplasmic membrane (28, 31).

If so, the defects observed in CcdA-deficient strains might be explained by inefficient disulfide bond isomerization in proteins localized on the outer surface of the membrane.

Some cortex or coat proteins and extracellular protein factors with a function in spore coat or cortex synthesis possibly contain one or more essential disulfide bonds (reference 9 and references therein). Efficient formation of these bonds and cross-linking of proteins in the coat could be mediated by the activity of CcdA. In the absence of CcdA, proteins with several cysteine residues and located on the outer surface of the membrane would be more commonly misfolded or only slowly folded into a functional state. The resulting small amounts of functional protein in the intermembrane space between the mother cell and the forespore might limit the synthesis of heat-resistant spores, which would be observed as a reduced sporulation efficiency compared to that of wild-type strains.

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REFERENCES

1. **Barker, P. D., and S. J. Ferguson.** 1999. Still a puzzle: why is haem covalently bound in *c*-type cytochromes? Structure **7:**R281–R290.
2. **Bengtsson, J., C. Rivolta, L. Hederstedt, and D. Karamata.** 1999. *Bacillus subtilis* contains two small *c*-type cytochromes with homologous heme-domains but different types of membrane-anchors. J. Biol. Chem. **274:**26179–26184.
3. **Bron, S.** 1990. Plasmids, p. 75–174. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biological methods for *Bacillus*. John Wiley & Sons, Inc., New York, N.Y.
4. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL1-Blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. BioTechniques **5:**376–379.
5. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, et al.** 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **393:**537–544.
6. **Crooke, H., and J. Cole.** 1995. The biogenesis of *c*-type cytochromes in *Escherichia coli* requires a membrane-bound protein, DipZ, with a protein disulphide isomerase domain. Mol. Microbiol. **15:**1139–1150.
7. **Dandekar, T., B. Snel, M. Huynen, and P. Bork.** 1998. Conservation of gene order: a fingerprint of proteins that physically interact. Trends Biochem. Sci. **23:**324–328.
8. **Deshmukh, M., G. Brasseur, and F. Daldal.** 2000. Novel *Rhodobacter capsulatus* genes required for the biogenesis of various *c*-type cytochromes. Mol. Microbiol. **35:**123–138.
9. **Driks, A.** 1999. *Bacillus subtilis* spore coat. Microbiol. Mol. Biol. Rev. **63:**1–20.
10. **Errington, J.** 1993. *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. Microbiol. Rev. **57:**1–33.
11. **Fortnagel, P., and E. Freese.** 1968. Analysis of sporulation mutants. II. Mutants blocked in the citric acid cycle. J. Bacteriol. **95:**1431–1438.
12. **Fridén, H., and L. Hederstedt.** 1990. Role of His residues in *Bacillus subtilis* cytochrome *b*558 for haem binding and assembly of succinate:quinone oxidoreductase (complex II). Mol. Microbiol. **4:**1045–1056.
13. **Green, G. N., and R. B. Gennis.** 1983. Isolation and characterization of an *Escherichia coli* mutant lacking cytochrome *d* terminal oxidase. J. Bacteriol. **154:**1269–1275.
14. **Guérot-Fleury, A. M., N. Frandsen, and P. Stragier.** 1996. Plasmids for ectopic integration in *Bacillus subtilis*. Gene **180:**57–61.
15. **Haima, P., S. Bron, and G. Venema.** 1987. The effect of restriction on shotgun cloning and plasmid stability in *Bacillus subtilis* Marburg. Mol. Gen. Genet. **209:**335–342.
16. **Harwood, C. R., and S. M. Cutting.** 1990. Molecular biological methods for *Bacillus*. John Wiley & Sons, Inc., New York, N.Y.
17. **Hederstedt, L.** 1986. Molecular properties, genetics, and biosynthesis of *Bacillus subtilis* succinate dehydrogenase complex. Methods Enzymol. **126:**399–414.
18. **Hoch, J. A.** 1991. Genetic analysis in *Bacillus subtilis*. Methods Enzymol. **204:**305–320.
19. **Kranz, R., R. Lill, B. Goldman, G. Bonnard, and S. Merchant.** 1998. Molecular mechanisms of cytochrome *c* biogenesis: three distinct systems. Mol. Microbiol. **29:**383–396.
20. **Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, et al.** 1997. The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. Nature **390:**249–256.
21. **Le Brun, N. E., J. Bengtson, and L. Hederstedt.** Genes for cytochrome *c* synthesis in *Bacillus subtilis*. Mol. Microbiol., in press.
22. **Madhusudan, J. Zapf, J. M. Whiteley, J. A. Hoch, N. H. Xuong, and K. I. Varughese.** 1996. Crystal structure of a phosphatase-resistant mutant of sporulation response regulator Spo0F from *Bacillus subtilis*. Structure **4:**679–690.
23. **Margolis, P., A. Driks, and R. Losick.** 1991. Establishment of a cell type by compartmentalized activation of a transcription factor. Science **254:**562–565.
24. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. **3:**208–218.
25. **Missiakas, D., F. Schwager, and S. Raina.** 1995. Identification and characterization of a new disulfide isomerase-like protein (DsbD) in *Escherichia coli*. EMBO J. **14:**3415–3424.
26. **Niaudet, B., A. Goze, and S. D. Ehrlich.** 1982. Insertional mutagenesis in *Bacillus subtilis*: mechanism and use in gene cloning. Gene **19:**277–284.
27. **Page, D. M., Y. Sambongi, and S. J. Ferguson.** 1998. Contrasting routes of *c*-type cytochrome assembly in mitochondria, chloroplasts and bacteria. Trends Biochem. Sci. **23:**103–108.
28. **Page, D. M., N. F. W. Saunders, and S. J. Ferguson.** 1997. Disruption of the *Pseudomonas aeruginosa dipZ* gene, encoding a putative protein-disulfide reductase, leads to partial pleiotropic deficiency in *c*-type cytochrome biogenesis. Microbiology **143:**3111–3122.
29. **Pettigrew, G. W., and G. R. Moore.** 1987. Cytochromes *c*: biological aspects. Springer Verlag KG, Berlin, Germany.
30. **Raleigh, E., and G. Wilson.** 1986. *Escherichia coli* K-12 restricts DNA containing 5-methylcytosine. Proc. Natl. Acad. Sci. USA **83:**9070–9074.
31. **Rietsch, A., D. Berlin, N. Martin, and J. Beckwith.** 1996. An *in vivo* pathway for disulfide bond isomerization in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **93:**13048–13053.
32. **Rosenkranz, M. S., D. W. Dingman, and A. L. Sonenshein.** 1985. *Bacillus subtilis citB* gene is regulated synergistically by glucose and glutamine. J. Bacteriol. **164:**155–164.
33. **Sambongi, Y., and S. J. Ferguson.** 1994. Specific thiol compounds complements the deficiency in *c*-type cytochrome biogenesis in *Escherichia coli* carrying a mutation in a membrane-bound disulphide isomerase-like protein. FEBS Lett. **353:**235–238.
34. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
35. **Schiött, T., M. Trone-Holst, and L. Hederstedt.** 1997. *Bacillus subtilis* CcdA-defective mutants are blocked in a late step of cytochrome *c* biogenesis. J. Bacteriol. **179:**4523–4529.
36. **Schiött, T., C. von Wachenfeldt, and L. Hederstedt.** 1997. Identification and characterization of the *ccdA* gene required for cytochrome *c* synthesis in *Bacillus subtilis*. J. Bacteriol. **179:**1962–1973.
37. **Schägger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166:**368–379.
38. **Spizizen, J.** 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc. Natl. Acad. Sci. USA **44:**1072–1078.
39. **Stewart, E. J., F. Katzen, and J. Beckwith.** 1999. Six conserved cysteines of the membrane protein DsbD are required for the transfer of electrons from the cytoplasm to the periplasm of *Escherichia coli*. EMBO J. **18:**5963–5971.
40. **Stock, A. M., J. M. Mottonen, J. B. Stock, and C. E. Schutt.** 1989. Three-dimensional structure of CheY, the response regulator of bacterial chemotaxis. Nature **337:**745–749.
41. **Stragier, P.** 1996. Molecular genetics of sporulation in *Bacillus subtilis*. Annu. Rev. Genet. **30:**297–341.
42. **Stragier, P., C. Bonamy, and C. Karmazyn-Campelli.** 1988. Processing of a sporulation sigma factor in *Bacillus subtilis*. How morphological structure could control gene expression. Cell **532:**697–704.
43. **Taber, H. W.** 1993. Respiratory chains, p. 199–212. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria. American Society for Microbiology, Washington, D.C.
44. **Thöny-Meyer, L.** 1997. Biogenesis of respiratory cytochromes in bacteria. Microbiol. Mol. Biol. Rev. **61:**337–376.
45. **Tochikubo, K.** 1971. Changes in terminal respiratory pathways of *Bacillus subtilis* during germination, outgrowth and vegetative growth. J. Bacteriol. **108:**652–661.
46. **van der Oost, J., C. von Wachenfeldt, L. Hederstedt, and M. Saraste.** 1991. *Bacillus subtilis* cytochrome oxidase mutants: biochemical analysis and genetic evidence for two aa_3-type oxidases. Mol. Microbiol. **5:**2063–2072.
47. **von Heijne, G.** 1992. Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. J. Mol. Biol. **225:**487–494.
48. **von Wachenfeldt, C., and L. Hederstedt.** 1990. *Bacillus subtilis* 13 kDa cytochrome *c*-550 encoded by *cccA*, consists of a membrane-anchor and a heme

domain. J. Biol. Chem. **265:**13939–13948.
49. **von Wachenfeldt, C., and L. Hederstedt.** 1992. Molecular biology of *Bacillus subtilis* cytochromes. FEMS Microbiol. Lett. **100:**91–100.
50. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
51. **Yu, J., L. Hederstedt, and P. Piggot.** 1995. The cytochrome *bc* complex (menaquinol:cytochrome *c* reductase) in *Bacillus subtilis* has a non-traditional subunit organization. J. Bacteriol. **177:**6751–6760.
52. **Yu, J., and N. E. Le Brun.** 1998. Studies of the cytochrome subunits of menaquinone:cytochrome *c* reductase (*bc* complex) of *Bacillus subtilis*. J. Biol. Chem. **273:**8860–8866.
53. **Zheng, L., and R. Losick.** 1990. Cascade regulation of spore coat gene expression in *Bacillus subtilis*. J. Mol. Biol. **212:**645–660.